University of Alberta

Neuroprotection by Retavase® and Integrilin® alone or in combination for the treatment of embolic middle cerebral artery occlusion

By

Asim Javed Raja

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Centre for Neuroscience

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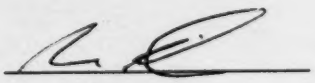

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Neuroprotection by Retavase and Integrilin Alone or in Combination for the Treatment of Embolic Middle Cerebral Artery Occlusion submitted by Asim Javed Raja in partial fulfillment of the requirements for the degree of Master of Science in Neuroscience.

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Abstract

Thrombolytic efficacy is limited by the dose-dependent risk of intracerebral hemorrhage. The combination of antiplatelet (Integrilin®) and thrombolytic (Retavase®) drugs may reduce the probability of intracerebral bleeding by lowering the dose of thrombolytic drug required to achieve reperfusion. This strategy was employed in a rat model of embolic middle cerebral artery occlusion.

Ischemic and control rats were divided into the following treatment groups: Retavase®, Retavase® + Integrilin®, Integrilin, and no treatment. Varying doses of Retavase® were evaluated in the Retavase® only and Retavase® + Integrilin® groups. Rats were sacrificed 72 hours post occlusion. The dependent variables of infarct size, neurological deficit score, incidence of hemorrhagic transformation, and mortality were determined and compared between groups.

Ischemic rats in the untreated group had the largest infarct volumes, neurological deficit scores, and mortality. Augmentation of rPA (Retavase®) therapy with Integrilin® allowed for a lower effective dose of rPA, associated with reduced incidence of hemorrhagic transformation while maintaining significant reductions in all other examined parameters.

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List of Abbreviations

5-HT = 5-hydroxytryptamine = serotonin
AA = arachidonic acid
ACA = anterior carotid artery
ANOVA = Analysis of Variance
ATP = adenosine triphosphate
BA = basilar artery
CCA = common carotid artery
e.g. = example
ECA = external carotid artery
H & E = hematoxylin and eosin
ICA = internal carotid artery
MI = myocardial infarction
MCA = middle cerebral artery
MCAo = middle cerebral artery occlusion
MRI = magnetic resonance imaging
ND = neurological deficit score
NMDA = N-methyl-D-aspartate
PCA = posterior communicating artery
PE = polyethylene
PgpA = pterygopalatine artery
r = right (eg. rMCA = right MCA)
rPA = recombinant plasminogen activator, i.e. Retavase® (reteplase)
rPgpA = right pterygopalatine artery
R = receptor (e.g. 5-HTR = 5-HT receptor)
TIMI = thrombosis in myocardial infarction
tPA = tissue plasminogen activator, i.e. Activase (alteplase)
TTC = 2,3,5-triphenyltetrazolium chloride
TXA_2 = thromboxane A_2
VA = vertebral artery

Chapter 1: Background

Stroke is the third leading cause of death and the leading cause of adult disability in the West, including Canada and the United States (Murphy, 2000). American studies on the cost of stroke estimate the annual bill at $41 billion U.S. (Taylor, Davis, Torner *et al.*, 1996; Schretzman, 1999). This dollar figure does not take into account the physical and psychological impact on the victims, who may be left unable to speak, move, or care for themselves. Neither does the economic estimate take into the account the hardships endured by the caregivers of stroke victims.

Besides testifying to the widespread prevalence of this devastating affliction, these statistics show that the currently available treatments for stroke are inadequate. Tissue plasminogen activator (tPA) is the only therapy for stroke approved by the Food and Drug Administration (Albers, 1999; Traynelis and Lipton, 2001). However, the risk of cerebral hemorrhage associated with tPA use means that only a small percentage (approximately 7%) of ischemic stroke patients qualify for this therapy. A dire need exists to develop more effective and inclusive methods of treating stroke.

Stroke compromises blood flow to the brain and can result from a number of conditions. Hemorrhagic stroke refers to bleeding in the brain and may be classified as subarachnoid hemorrhage or intracerebral hemorrhage. Subarachnoid hemorrhage results from bleeding in the subarachnoid space (beneath the dura mater), whereas intracerebral hemorrhage is caused by bleeding of the intracranial blood vessels, resulting in leakage of blood inside the brain tissue itself.

Ischemic stroke occurs when blood flow to the brain is reduced. Focal ischemia results from occlusion of a cerebral artery by a clot whereas global ischemia is caused by

a decrease or cessation of blood flow to the entire brain, such as takes place during cardiac arrest. These are two very different conditions.

Global ischemia, if not corrected, results in death within minutes. Transient global ischemia, which is a brief cessation of blood flow to the entire brain, results in different effects on different areas of the brain. The most sensitive region of the brain is the hippocampus (Bottiger, Teschendorf, Krumnikl *et al.*, 1999; Ferrand-Drake, 2001). This area (the CA1 neurons) experiences selective neuronal damage during transient global ischemia accompanied by activation of microglia and the resulting release of a variety of cytokines (Sairanen, Lindsberg, Brenner *et al.*, 1997; Lin, Ginsberg, Busto *et al.*, 1998). Treatment options being explored for global ischemia include neuroprotective drugs (Artemenko, Gerasimov, Krishtal, 2000; Kuhmonen, Jolkkonen, Haapalinna *et al.*, 2000) that aim to antagonize the harmful changes in neuronal biochemistry induced by the ischemia as well as anti-inflammatory agents (Dietrich, Busto, Bethea, 1999).

Focal ischemia results in decreased blood flow to those areas of the brain irrigated by the occluded artery. Although a cerebral artery may irrigate a large portion of the brain, collateral circulation allows for blood to be received by more than one artery. This results in a blood flow gradient with lowest blood flow nearest to the occlusion site and increasingly greater blood flow values further away from the site of occlusion (Heiss, Huber, Fink *et al.*, 1992). This is illustrated in figure 1.1. Areas with lowest blood flow (<10mL/100g/min) are most susceptible to ischemic damage and begin to die within minutes whereas those with moderately low blood flow (the penumbra, 15-30ml/100g/min) may survive for hours allowing an opportunity for rescue. Areas with slightly decreased blood flow (30 - 50ml/100g/min) may survive indefinitely as the ischemic tissue can compensate for decreased blood flow – this will be described in more

detail under *Cerebral artery occlusion*. Variable neuronal survival times were observed in a pig model of focal ischemia and were accounted for by variable rates of cerebral blood flow (Sakoh, Ostergaard, Rohl *et al.*, 2000).

Figure 1.1: Cerebral blood flow rates after embolization

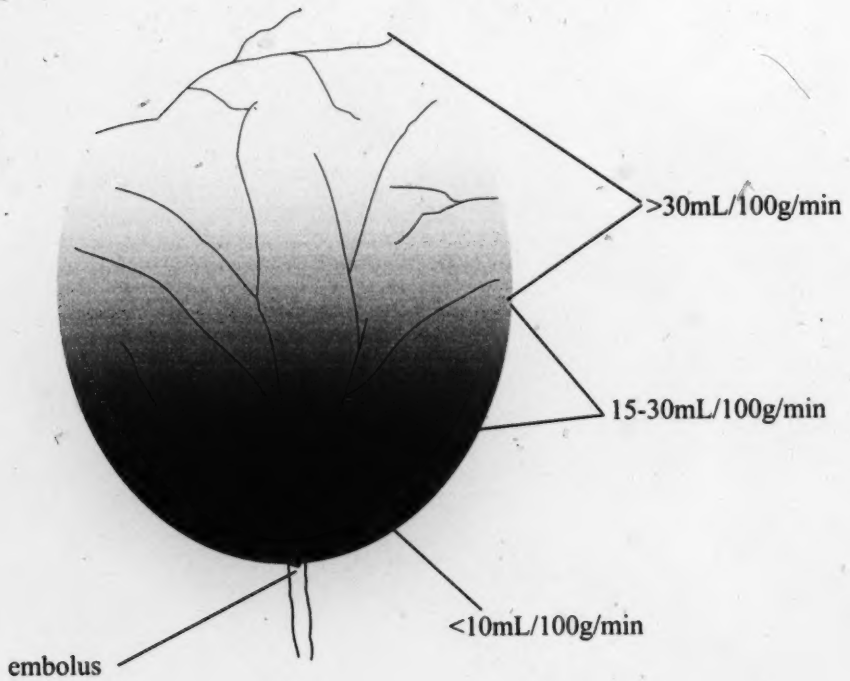

Figure 1.1
When a cerebral artery is blocked by an embolus, the greatest decrease in blood-flow occurs in the area nearest to the occlusion. Blood flow increases in a gradient-like fashion when measured further and further away from the embolus because of compensation from collateral circulation.
The penumbra, the target of therapeutic intervention, is the region of brain tissue receiving moderate blood flow (approximately 15 to 30 mL/100g/min). This region may survive for hours, providing a window of opportunity for rescue.

Cerebral artery occlusion

Sometimes blood clots end up in places other than that of vessel injury – the result may be life-threatening. A thrombus is a blood clot that blocks an artery (e.g. coronary or cerebral). A thromboembolus is a fragment of a thrombus that blocks an artery downstream from the thrombus.

Healthy brain tissue receives a steady blood supply averaging 50ml per 100g of brain tissue per minute (Schmidt, Waldemar, Paulson, 1991). Cerebral artery occlusion results in a decrease of perfusion pressure (Kishi, Kawaguchi, Kurehara *et al.*, 2000), pH (Kajita, Takayasu, Dietrich *et al.*, 1998), and blood oxygen content (Brown, Wade, Marshall, 1985) and an increase in carbon dioxide (Morita, Hardebo, Bouskela, 1994) content downstream from the occlusion. All of these factors contribute to vasodilation. If vasodilation cannot compensate for the decreased blood pressure, the blood flow decreases and the oxygen extraction fraction (extraction of oxygen from the blood) increases (Frizzell, Meyer, Borchers *et al.*, 1991). If the neurons are still deprived of oxygen, adenosine triphosphate (ATP) levels are maintained by increasing glycolysis (Frizzell, Meyer, Borchers *et al.*, 1994). Where blood pressure is sufficiently compromised by the occlusion, all of these compensatory mechanisms put together are unable to maintain neuronal function and neurological deficits begin to appear.

This happens when blood flow is reduced to approximately 15mL/100g/min. At this point, the ability of neurons to fire action potentials is compromised, and thus neurological deficits appear (Jones, Morawetz, Crowell *et al.*, 1991) along with the slowing of the electroencephalogram (Sharbrough, Messick, Sundt, 1973), attenuation of evoked potential (Back,1998), and a decrease in synaptic potentials observed in single neurons (Heiss, Hayakawa, Waltz, 1976). ATP content may be normal or only slightly

below normal, and the physiological membrane potential is still intact (Naritomi, Sasaki, Kanashiro *et al.*, 1988), so the reason for this 'electrical failure' is not fully understood. In addition, extracellular levels of excitatory amino acids (glutamate and aspartate) begin to rise (Shimada, Graf, Rosner *et al.*, 1989).

Further reduction in blood flow causes glycolysis to cease and ATP levels diminish (Swanson, Farrell, Stein, 1997). Membrane gradients cannot be maintained and so the neurons depolarize and fire erratically. This is thought to cause lethal waves of 'electrical spreading depression' (Nielsen, Fabricius, Lauritzen, 2000). This spreading wave of depolarization is thought to contribute to ischemic excitotoxicity. Excitotoxicity is due primarily to Ca^{2+} overload. Excitatory amino acids that are released by depolarized ischemic neurons allow for Ca^{2+} entry via voltage-gated or ligand-gated channels, in particular the N-methyl-D-aspartate (NMDA) activated channel (Rothman and Olney, 1986). This, combined with the inability of neurons to extrude intracellular Ca^{2+} ions (lack of ATP), allows Ca^{2+} levels to reach toxic levels within neurons (Choi and Rotham, 1990; Katsura Kristian and Siesjo, 1994).

Ca^{2+} is involved in the activation of numerous enzymes and is therefore the key to initiating a multitude of biochemical pathways. For this reason, intracellular Ca^{2+} levels are tightly controlled. Increases in Ca^{2+} concentrations that take place during normal conditions are very brief in time and occur in a very small portion of the cytoplasmic space. The cytotoxic increase in Ca^{2+} observed in ischemic neurons results in the inappropriate activation of Ca^{2+}-dependent processes. While most of these processes are not toxic *per se*, and end up doing little more than wasting cellular resources, other Ca^{2+}-activated processes contribute to cell death via necrosis or apoptosis.

Ca^{2+} activates proteolytic enzymes such as calpains. Calpains catalyze the breakdown of vital cellular components, including the cytoskeleton (Minger, Geddes, Holtz *et al.*, 1998). They also activate caspases (Blomgren, Zhu, Wang *et al.*, 2001), which stimulate apoptosis (Seshagiri and Miller, 1997).

Lipolytic enzymes like Ca^{2+}-dependent phospholipase A_2 are also activated by the high intracellular Ca^{2+} levels in ischemic neurons. These enzymes degrade the phospholipid cell membrane and increase the intracellular free fatty acid concentrations by up to 20x (Gardiner, Nilsson, Rehncrona *et al.*, 1981). Degradation of membrane phospholipids as well as membrane instability due to rising fatty acid levels contributes to disintegration of the cell membranes.

Arachidonic acid is the primary free fatty acid released by lipolysis during ischemia (Gardiner *et al.*, 1991). During reperfusion, when oxygen becomes available to the neurons, arachidonic acid becomes a substrate for the cyclo-oxygenase and lipoxygenase pathways (Wieloch and Siesjo, 1982). Cyclooxygenase-2 metabolizes arachidonic acid into prostaglandins, producing free radical species in the process (Strauss, Barbe, Marshall *et al.*, 2000). Free radical species are extremely reactive and will react with and damage just about any molecule. These include membrane phospholipids, cellular proteins, and even DNA molecules. They play a role in apoptotic cell death (Kondo, Reaume, Huang *et al.*, 1997), probably by damaging the mitochondria, causing them to release cytochrome c, which stimulates apoptosis (Fujimura, Morita-Fujimura, Murakami *et al.*, 1998). The lipoxygenase enzymes metabolize arachidonic acid into leukotrienes (Ohtsuki, Matsumoto, Hayashi *et al.*, 1995). Almost all leukotrienes are vasoconstrictors (Uski and Hogestatt, 1992) and they contribute to cerebral edema (Mabe, Nagai, Suzuka, 1990). This is likely due to disruption of the

blood-brain barrier (Rao, Hatcher, Kindy *et al.*, 1999). Edema is the primary cause of death in stroke patients (Schwab, Schwarz, Spranger *et al.*, 1998; Berrouschot, Rossler, Koster *et al.*, 2000).

Leukotriene disruption of the blood-brain barrier is likely due to the fact that leukotrienes serve as chemoattractants to leukocytes, stimulating their adhesion to the vascular endothelium and migration through the blood-brain barrier into the neuronal tissue (Aktan, Aykut, Oktay *et al.*, 1992; Dorovini-Zis, Bowman, Prameya, 1992). It is thought that leukocyte adhesion may occlude small cerebral arteries and disrupt the blood-brain barrier. Ischemia is also correlated with microglial activation which releases inflammatory cytokines, many of which are responsible for neuronal damage (Saito, Suyama, Nishida *et al.*, 1996; Flavin and Ho, 1999).

The region of brain tissue surrounding the ischemic core that receives moderate blood blow (more than 15mL/100g/min) is termed the penumbra. These cells contribute to neurological deficit but are potentially viable as they do not degenerate immediately. Thus a window of opportunity exists to salvage this tissue, and the treatments for ischemic stroke center around preventing the penumbra from degenerating to infarction.

The blood clotting system

The blood's ability to clot allows punctures of blood vessels to be sealed, preventing excessive bleeding and preserving the blood supply. As such the clotting system is designed to be triggered by damage to the vasculature. After vascular damage, a cascade of reactions, including activation of a variety of proteins, results in the formation of a clot. A detailed description of clotting reactions may be found in Chapter 2 of Molecular Basis of Thrombosis and Hemostasis (High and Roberts, 1995).

Tissue factor is a protein expressed at all times in the blood vessel adventicia. Thus, breaking of the vessel wall causes tissue factor to be released into the surrounding blood, where it binds to factor VII. This complex, after becoming activated (mechanism unknown), activates factors IX and X. These activated factors cause the conversion of small amounts of prothrombin to thrombin, which activates platelet cells – these cells are instrumental in clot formation. It is not fully known how the platelets aggregate in the vicinity of vessel injury. Broken vessel walls express Von Willebrand factor which binds to the platelet glycoprotein GPIb. It is important to note that there are many activators of platelets (e.g. Thromboxane A_2), but during vessel-injury mediated clotting, thrombin is the important activator. An inhibitor of any of the platelet activators can be classified as an antiplatelet drug.

'Tenase complexes' and 'prothrombinase complexes' can only form on the surface of activated platelets. These complexes are formed by co-localization of activated blood factors on the platelet cell membrane. The 'tenase complex' allows for the rapid activation of factor X, on a scale much greater than that achieved by the tissue factor – factor VII complex. Activated factor X forms the prothrombinase complex with activated factor V. This allows for very rapid conversion of prothrombin to thrombin. Activated platelets also express the platelet membrane glycoprotein GPIIb/IIIa, a protein not expressed by non-activated platelets. This glycoprotein binds to fibrinogen which is normally found in the blood. Fibrinogen's divalent structure allows for platelet aggregation by the bridging GPIIb/IIIa molecules from adjacent platelets (with fibrinogen as the bridge). At this point, the aggregate is still permeable to blood. Thrombin causes the conversion of fibrinogen to fibrin, resulting in a visible fibrin clot. Activated factor VIII (part of the tenase complex) cross-links the fibrin molecules, making the clot

impermeable to blood, allowing it to seal the puncture. This sequence of steps has been summarized in figure 1.2.

Figure 1.2: Major reactions involved in blood clotting

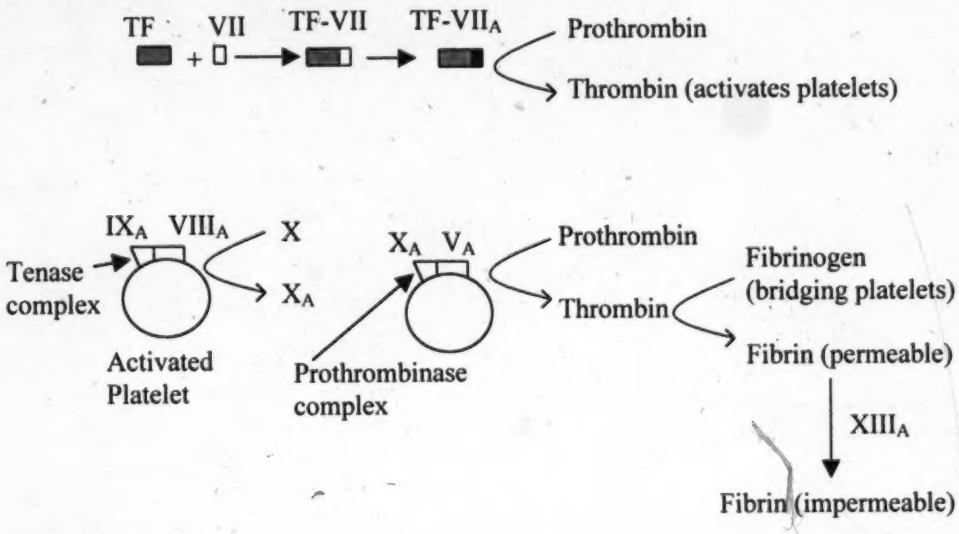

Figure 1.2
Tissue factor (TF), released from damaged vascular endothelium, binds to factor VII and causes its activation. This activated complex converts prothrombin to its active form, thrombin which in relatively low concentrations activates platelet cells (fig 1.4).
The Tenase complex, comprised of activated factors IX and VIII, activates factor X which, along with activated factor V, comprises the prothrombinase complex.
The prothrombinase complex converts prothrombin to thrombin on a much larger scale than the tissue factor/factor VII$_A$ complex. Higher concentrations of thrombin convert fibrinogen (a protein that bridges platelets) into fibrin, which is further modified by activated factor XIII.
The resulting impermeable platelet plug serves to seal the injured vascular endothelium, preventing loss of blood from that site.

Adapted from Molecular Basis of Thrombosis and Hemostatis (High and Roberts, 1995)

Rat models of middle cerebral artery occlusion

To test the potential efficacy of any drug on stroke patients, an animal model of stroke must be used. Ideal models closely resemble clinical situations. When compared to gerbils, cats, and dogs, the rat cranial circulation is most similar to that of the human (Yamori, Horie, Handa *et al.,* 1976). A rat model of MCA occlusion has been used very extensively and successfully in our lab, and so this model was selected for this study.

The MCA is believed to be the most commonly blocked artery in embolic stroke (Karpiak, Tagliavia, Wakade, 1989). Thus the ideal model for this study would be a rat model of MCA occlusion. However, there are many models of MCA occlusion in the rat.

Some models involve permanent occlusion of the MCA. An example is the Tamura model (Tamura, Graham, McCulloch *et al.,* 1981), where the MCA is exposed and occluded. How the occlusive material was introduced or what the material is was not specified by the authors. Their diagram showed what resembled a 2-3mm long filament inserted into the origin of the MCA. Unfortunately, permanent MCA occlusion (MCAo) does not accurately mimic the typical clinical situation. Spontaneous recanalization occurs in up to 50% of cases involving stroke patients suffering from MCA occlusion (Saito, Segawa, Shiokawa *et al.,* 1987). A model that allows reperfusion to take place would thus be more useful.

The most popular model in use today is the intraluminal filament model, first described by Koizumi and colleagues (Koizumi, Yoshida, Nakazawa *et al.,* 1986). In this model, a nylon filament (coated with poly-L-lysine) is advanced up the internal carotid artery all the way up to a few millimeters beyond the MCA (figure 1.3). Infarction size can be controlled by the length of time the nylon thread is left inside. Another rat model of reversible MCAo is prepared by application of endothelin-1 (a very potent

vasoconstrictor) to the exposed MCA (Robinson, Macrae, Todd *et al.*, 1990). Endothelin causes vasoconstriction of the MCA for a period of time, followed by relaxation allowing for reperfusion. The amount of endothelin applied affects the strength and duration of MCA vasoconstriction, and thereby affects the severity of infarction. Other methods of inducing ischemia followed by reperfusion involve the temporary occlusion of arteries using surgical clips (Dietrish, Nakayama, Watson *et al.*, 1989), hooks (Kaplan, Brint, Tanabe *et al.*, 1991) or ligature snares (Shigeno, Teasdale, McCulloch *et al.*, 1985).

While it is useful to be able to control the severity of infarction and avoid mechanical damage to the vessels and the brain itself, these models are limited to the study of mechanisms of cell death, neuroprotective agents, or any other study not requiring the presence of a clot. Thrombolytic and antiplatelet agents cannot be studied using this model, as their efficacy arises from their ability to disintegrate a clot and allow for reperfusion. Also, it has been observed that occlusion of the MCA by a platelet thrombus produces more serious consequences for ischemic cells than occlusion by a surgical clip, even though the occlusion was maintained for the same time period and complete reperfusion was attained afterward (Dietrich, Nakayama, Watson *et al.*, 1989). This was probably due to biochemical changes induced in the blood by the clot that were not mimicked by the surgical clip.

Figure 1.3: Intraluminal filament model

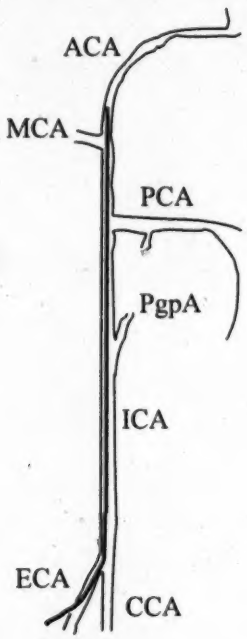

Figure 1.3
A nylon filament extends past the middle cerebral artery (MCA) compromising blood flow to the brain areas irrigated by the right middle cerebral artery. Note that the nylon filament prevents blood from the common carotid artery (CCA), anterior cerebral artery (ACA) and the posterior communicating artery (PCA) from entering the middle cerebral artery.

For this experiment, a rat model of MCAo required the MCA to be occluded by a clot. Besides more closely resembling the clinical situation than the models described thus far, this model will allow for the study of the drugs to be used in this experiment. A number of these 'thromboembolic models' exist.

The photochemical models (Watson, Dietrich, Busto *et al.*, 1985) work by application of a specific wavelength of light over an area of the rat's skull during intravenous infusion with a photosensitive dye. The dye reacts with the light to produce oxygen radicals which peroxidize endothelial lipids (i.e. damage them and stimulate the clotting reactions in those areas), triggering thrombosis. When performed correctly, this method produces very consistent infarcts (Nakase, Kempski, Heimann *et al.*, 1997). Unlike the other occlusion models, the time course of blood-brain barrier disruption is very different from that in human cerebral ischemia (Forsting, Reith, Dorfler *et al.*, 1994). Therefore the model has limited validity in focal ischemia research.

Another model that has been described involves injection of crushed human blood clot fragments (about 100μm - 300μm after crushing) into the internal carotid artery (Papadopoulos, Chandler, Salamat *et al.*, 1987). Although this results in actual emboli that block cerebral arteries, and the MCA is very likely to be blocked in these models, the final placement of the emboli is difficult to control for. Therefore the location and size of the infarct varies with these models.

This shortcoming was effectively tackled by the development of a model in which thrombin (blood clotting agent) was injected at the base of the MCA itself. The laboratory that developed this similar model demonstrated with magnetic resonance imaging (MRI) that thrombin injection into the MCA was followed by reduced cerebral blood flow in the affected hemisphere (Zhang, Zhang, Jiang *et al.*, 1997). Administration

of tissue plasminogen activator restored blood-flow. Post-mortem analysis of the rat brains revealed fibrin-rich clots at the base of the MCA and infarcts that were consistent in location and volume. The size of the infarct was controlled for by the amount of thrombin injected.

The MCAo model I used entails injection of a preformed thrombus into the base of the MCA (Wang, Yang, Yang, Shuaib, 2001). Not only does this model employ induction of cerebral ischemia with a preformed clot, the placement of the clot is at the base of the MCA itself. This minimizes the variation in infarct size and ensures that the same brain area is affected in all the experimental rats. The size of the clot (controlled by the volume of blood used to make the clot) injected affects the severity of infarction.

This model is advantageous over the Zhang model because the surgical procedure is simpler and less traumatic for the rats, the size of the clot is more accurately controlled, and there is no possibility of thrombin being carried by the blood to other areas of the brain. For these reasons, this model is used in our laboratory to study the potential value of drug treatments for stroke patients. The procedure for preparing this model is described in detail in Chapter 2: Methods, under the section, "Introduction of an embolus into the MCA".

Neuroprotective therapy

Neuroprotective therapies aim to halt or reverse the detrimental biochemical processes that are activated in neurons by ischemia. As there are many processes that are stimulated by ischemia, there are also a whole host of neuroprotective drugs. NMDA antagonists, such as the magnesium ion, prevent activation of the NMDA receptor itself and should therefore inhibit all the lethal cascades that follow excessive NMDA

stimulation during ischemia. A 5% magnesium sulfate solution was able to reduce infarct sizes in rats up to 6hrs after embolic MCA occlusion (Yang, Li, Ahmed *et al.*, 2000). Thus the window of opportunity for magnesium therapy is longer than for thrombolytic therapy.

Neuroprotection has also been observed in animal models of cerebral ischemia with free radical scavengers (Yang, Li, Shuaib, 2000), GABA agonists (Kume, Greenfield, Macdonald *et al.*, 1996; Chen, Yi, Qui *et al.*, 2000), sodium channel blockers (Shuaib, Mahmood, Wishart *et al.*, 1995), Ca^{2+} channel blockers (Mohamed, Gotoh, Graham *et al.*, 1985; Deshpande and Wieloch, 1986), protein synthesis inhibitors (Linnik, Zobrist, Hatfield, 1993), calpain inhibitors (Li, Howlett, He *et al.*, 1998), and cytokine inhibitors (Flavin and Ho, 1999). This is not an exhaustive list - these drugs are tested most often in animal models as their effects on ischemic neurons are best understood. In addition, many of these drugs are being tested in combination with thrombolytics and other neuroprotective therapies.

Unfortunately, none of these neuroprotective therapies has shown benefit in clinical trials (Kobayashi and Mori, 1998; Davalos, 1999). It is thought that the side effects of these medications manifest themselves at concentrations lower than those required for therapeutic benefit. In addition, it is easier to achieve statistical significance in animal experiments where the lesion size and location can be controlled. It is hoped that neuroprotective therapies with wider therapeutic indices may show benefit in stroke patients.

Hypothermia, which has also demonstrated neuroprotection in rat models of MCA, occlusion is thought to achieve its benefits through a variety of mechanisms, like reducing metabolic rate (Kawamura, Suzuki, Hadeishi *et al.*, 2000), lowering the levels

of excitatory amino acids (Li, He Miyashita *et al.*, 1999), lowering the concentrations of oxygen free radicals (Zhao, Richardson, Mombourquette *et al.*, 1996) in ischemic neurons and decreasing the severity of edema (Kawai, Nakamura, Okauchi *et al.*, 2000). A retrospective study of patients with acute stroke showed that mortality was higher in patients with hyperthermia and lower in patients with hypothermia (Wang, Lim, Levi *et al.*, 2000). Clinical trials assessing the effects of hypothermia on patients suffering acute stroke are therefore warranted.

Thrombolytic therapy

Thrombolytic therapies aim to disintegrate the emboli responsible for cerebral artery occlusion, thus restoring blood flow to ischemic brain regions. Presently, tissue plasminogen activator (tPA) is the only approved thrombolytic therapy for stroke (Benavente and Hart, 1999). This protein converts the inactive enzyme, plasminogen, to plasmin. Plasmin disintegrates fibrin, the principal component of an embolus. Administration of tPA therefore results in destruction of emboli and resumption of blood flow through occluded arteries. Although other promising treatments exist, safety issues need to be settled through completion of clinical trials before they can be approved for use by the medical community.

The effectiveness of tPA treatment has been demonstrated by the well known clinical trials held by The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group (NINDS, 1995). The major drawback to tPA treatment is the dose-dependent risk of cerebral hemorrhage associated with its use. This risk eliminates any benefit if tPA is administered more than three hours after stroke onset (Hacke, Kaste, Fieschi *et al.*, 1995). For a patient to be eligible for tPA treatment, stroke onset must be

less than three hours prior to tPA administration and there must be no evidence of intracranial bleeding. There are numerous other exclusion criteria, including age, history of bleeding, history of anticoagulant consumption, and blood levels of platelets and glucose (Adams, Brott, Furlan *et al.*, 1996). Due to the stringent screening criteria, only a fraction of stroke patients qualify for tPA treatment, and of those who qualify, one in fifteen can still be expected to experience massive brain hemorrhage (NINDS, 1995). These restrictions diminish the practicality and effectiveness of the use of tPA and provide justification for research of alternative or supplementary therapies.

When an artery is occluded, not only is neural tissue affected, the vasculature (including the blood-brain barrier) also undergoes degenerative changes (Hamann, Okada, Fitridge *et al.*, 1995; del Zoppo, Haring, Tagaya, 1996; Wagner, Tagaya, Koziol *et al.*, 1997; Fujimura, Gasche, Morita-Fujimura *et al.*, 1999). These changes explain the observed increase in vascular permeability as measured by a protein tracer (Dietrich, Busto, Halley *et al.*, 1990) and Evan's blue dye (Belayev, Busto, Ikeda *et al.*, 1998). It has been suggested that increased vascular permeability is one of the reasons why thrombolytic treatment can result in hemorrhagic transformation (del Zoppo, von Kummer, Hamann, 1998). As mentioned, tPA converts plasminogen to plasmin. It has been shown that plasmin cleaves laminin (Liotta, Goldfarb, Brundage *et al.*, 1981; Liotta, Goldfarb, Terranova, 1981), an integral component of the basal lamina (part of the permeability barrier). Thus, tPA degrades the blood-brain barrier that has already become vulnerable due to ischemia. For this reason, supplementary therapy (that itself does not contribute to vascular damage) that would decrease the dose of tPA required to achieve clot lysis is expected to decrease the risk of intracerebral hemorrhage.

Retavase® is a recombinant plasminogen activator (rPA). It is a modified tPA

protein (deletion mutant) expressed by *E. Coli*. The Retavase® gene lacks the epidermal growth factor (EGF) domain, the Kringle 1 domain, and the finger domain and the oligosaccharide side chains (due to expression in *E. Coli*) while maintaining the serine protease domain (responsible for thrombolytic effect) of the tPA protein (Martin, Bader, Böhm *et al.*, 1993). The EGF and Kringle 1 domains bind to receptors in the liver, allowing for their clearance (Martin, Fischer, Kohnert *et al.*, 1991). This is the likely reason why rPA has a longer half-life than tPA (Martin *et al.*, 1991). The longer half-life means that rPA can be administered as a double bolus, where as tPA must be infused (Gulba, Tanswell, Dechend *et al.*, 1997).

Retavase® has many other advantages over tPA. In patients with myocardial infarction (MI), rPA restored coronary blood flow more quickly than tPA, and unlike tPA, the dose of rPA does not have to be adjusted to the patient's weight (Smalling, Bode, Kalbfleisch *et al.*, 1995; Bode, Smalling, Berg *et al.*, 1996). It is as safe as tPA (Smalling *et al.*, 1995) and unlike some thrombolytics (e.g. Streptokinase), it does not induce an immune reaction (Martin *et al.*, 1991). In summary, rPA is a better thrombolytic in MI patients because it can be administered in a fast and convenient manner, it is a more powerful thrombolytic, and it has a safety profile comparable to that of tPA. Studies examining the efficacy of Retavase® treatment for embolic stroke have not been published.

Antiplatelet therapy

Antiplatelet drugs prevent the aggregation of platelets (essential for clot formation). There are many pathways that lead to platelet activation (figure 1.4), thus a number of antiplatelet drugs with various mechanisms of action exist.

Figure 1.4: Platelet activation

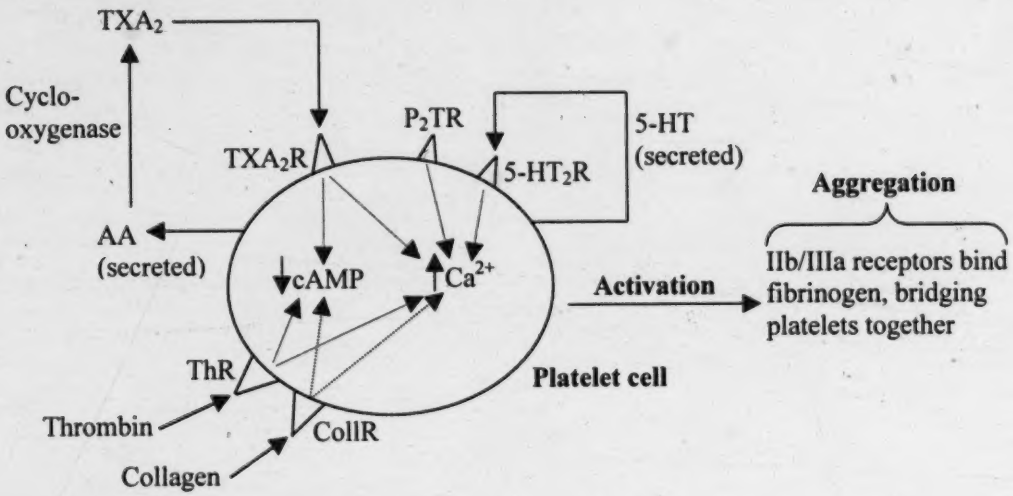

Figure 1.4
Platelet cell membranes contain receptors for thrombin, thromboxane A_2 (TXA_2), serotonin (5-HT), and ADP (P_{2T} receptor). A decrease in cAMP and/or increase in Ca^{2+} causes platelet activation (i.e. shape change, activated tenase complex, GP IIb/IIIa expression, etc.). Thrombin or collagen receptor stimulation results in decreased cAMP, increased Ca^{2+} and secretion of 5-HT and arachidonic acid (AA), which is convereted to TXA_2 by cyclooxygenase. Stimulation of the TXA_2 and P_{2T} receptors decreases cAMP and increases Ca^{2+}. Stimulation of the 5-HT_2 receptor increases Ca^{2+}. Therefore, stimulation of any of these receptors contributes to activating the platelet by altering levels of the second messengers cAMP and Ca^{2+}.

A platelet cell becomes activated after experiencing an intracellular increase of Ca^{2+} ion and/or decrease of intracellular cAMP – these second messengers appear to act independently of each other to activate the platelet cell (Pannocchia and Hardisty, 1985). Thrombin acts on its receptor (on the platelet surface) by cleaving it near the N-terminal. The new N-terminal that results from this cleavage acts as a ligand (tethered ligand) and activates the receptor (Vu, Hung, Wheaton *et al.*, 1990). This results in increased levels of intracellular Ca^{2+} ion (Vassallo, Kieber-Emmons, Cichowski *et al.*, 1992) and decreased levels of cAMP (Seiler, Michel, and Fenton, 1992; Vassallo *et al.*, 1992) due, at least in part, to G_i activation (Jakobs and Grandt, 1988; Ellis, Malik, Gilchrist *et al.*, 1999).

Platelets stimulated by thrombin or collagen (or a number of exogenous platelet activators) secrete arachidonic acid (AA) and serotonin (5-HT) (Yoshida and Aoki, 1978; Best, Holland, Jones *et al.*, 1980; Nosal', Jancinova, Danihelova, 2000). AA is converted to thromboxane A_2 by the enzyme cyclo-oxygenase. Platelet cell membranes contain receptors for both thromboxane A_2 and 5-HT. Activation of either (or both) of these receptors contributes to platelet activation. Stimulation of the thromboxane A_2 receptors causes an increase in intracellular Ca^{2+} (via release from intracellular stores) and a decrease in intracellular cAMP levels (Yoshida and Aoki, 1978; Soslau, McKenzie, Brodsky *et al.*, 1995). Stimulation of the 5-HT_2 receptor (by 5-HT) results in increased intracellular Ca^{2+} concentration (via release from intracellular stores) in the platelet cell (Erne and Pletscher, 1985; Nishio, Ikegami, Segawa, 1991; Roevens, De Clerck, de Chaffoy de Courcelles, 1993). As mentioned, an increase in intracellular Ca^{2+} and/or decrease in intracellular cAMP contributes to platelet activation (Pannocchia and Hardisty, 1985).

Antiplatelet medications prevent platelet aggregation by interfering with one or more of the processes necessary to change the platelet from an unactivated to activated or aggregated state. For example, aspirin inhibits the enzyme cyclo-oxygenase (Dejana, Cerletti, de Castellarnau *et al.,* 1981), preventing the conversion of AA (secreted by the platelet when it is stimulated by an activating agent) to thromboxane A_2 (Roth, Stanford, Majerus, 1975; Egan, Paxton, Kuehl, 1976). Likewise, 5-HT_2 receptor antagonists prevent 5-HT from activating platelet 5-HT_2 receptors. It has also been observed that P_{2T} receptor antagonists (e.g. ticlopidine and clopidogrel) demonstrate antiplatelet activity (Defreyn, Gachet, Savi *et al.,* 1991; Mills, Puri, Hu *et al.,* 1992). The P_{2T} (purinergic) receptors on the platelet cell membrane are activated by ADP. Activation of P_{2T} receptors by ADP (or any P_{2T} agonist) causes the opening of P_{2T} receptor-operated Ca^{2+} channels (Sage and Rink, 1987; Sage, Merritt, Hallam *et al.,* 1989; Hall and Hourani, 1993), allowing for an inward Ca^{2+} current and inhibition of adenylate cylcase (Cristalli and Mills, 1993). This results in reduced levels of cAMP (Mills, Figures, Scearce *et al.,* 1985). Thus, stimulation of P_{2T} receptors results in platelet aggregation (Hourani, Hall, Nieman, 1992; Humphries, Tomlinson, Ingall *et al.,* 1994; Ingall, Dixon, Bailey *et al.,* 1999). Antiplatelet medications need not act by affecting platelet receptors; some affect second messenger levels directly. For example, dipyridamole, a phosphodiesterase inhibitor, increases cyclic AMP levels by inhibiting the enzyme phosphodiesterase, which metabolizes cAMP (Dembinska-Kiec, Rucker, Schonhofer, 1979; Harker and Kadatz, 1983).

As there are many paths to activation of the platelets and many medications each with differing mechanisms of inhibiting platelet activation, one would expect that combining antiplatelet medications with differing modes of action would be more

effective at preventing platelet activation than any (single) antiplatelet drug. Such combination effects have been observed in numerous studies that have compared combinations of antiplatelet drugs (with differing mechanisms of action) with antiplatelet monotherapies. Many of these studies have looked at the ability of a single antiplatelet medication vs. a combination of such medications as adjuncts to thrombolytics. For example, the combination of an inhibitor of thromboxane synthase and a 5-HT_2 receptor antagonist proved to be a better adjunct to tPA in dogs than either aspirin or a 5-HT_2 receptor antagonist alone (McAuliffe, Moors, Jones, 1994). The European Stroke Prevention Study 2 (ESPS-2) showed that a combination of aspirin and dipyridamole provided additive benefit in stroke patients (Diener, Cunha, Forbes *et al.*, 1996). Clopidogrel in combination with aspirin was better as an adjunctive therapy to streptokinase than either aspirin or clopidogrel alone in a rabbit model of peripheral thrombosis (Herbert, Bernat, Sainte-Marie *et al.*, 1993).

With this reasoning, it would appear that the ideal antiplatelet medication should be able to inhibit all the pathways to platelet activation. Glycoprotein IIb/IIIa antagonists are a new class of antiplatelet medication; they prevent platelet aggregation by binding to the IIb/IIIa glycoprotein on the platelet cell membrane. Thus these drugs act by inhibiting the final common pathway to platelet aggregation (see figure 1.4). They are therefore much more effective than other drugs that inhibit platelet aggregation merely by blocking a single pathway to platelet activation. As expected, GPIIb/IIIa antagonists have demonstrated greater efficacy than aspirin (the most widely available antiplatelet drug) as an adjunct therapy to tissue plasminogen activator in canine models of coronary artery occlusion (Yasuda, Gold, Fallon *et al.*, 1988) and in preventing thrombosis and re-thrombosis in a canine carotid artery (Rote, Werns, Davis *et al.*, 1993). For this reason,

an affordable glycoprotein IIb/IIIa receptor antagonist, Integrilin®, was chosen for this study as an adjunct to Retavase®.

Like Retavase®, all Integrilin® studies have been done on patients with MI. Integrilin® (or eptifibatide) has a better safety profile (i.e. less bleeding) than ReoPro (or abciximab) because whereas ReoPro is an antibody medication that binds irreversibly to the platelet glycoprotein IIb/IIIa receptor, Integrilin® is a small peptide that binds competitively and reversibly to the glycoprotein IIb/IIIa receptor (Tcheng, 1996). In the event of bleeding complications and cessation of infusion of Integrilin®, normalization of bleeding time may be expected to take 4-6hrs (Harrington, Kleiman, Kottke-Marchant *et al.*, 1995; Integrilin® Product Monograph, 2000), whereas at least 24hrs are required for restoration of platelet function when ReoPro is administered (Tcheng, Ellis, George *et al.*, 1994; ReoPro® Product Monograph, 2000). In addition, the relatively small size of the Integrilin® molecule is probably the reason why there is no immune response to Integrilin® (Phillips and Scarborough, 1997), whereas an immune response is observed with administration of ReoPro (ReoPro® Product Monograph, 2000).

A number of studies, small and large, have demonstrated the effectiveness of eptifibatide treatment for MI. The original IMPACT (Integrilin® to Minimize Platelet Aggregation and Coronary Thrombosis) trial observed that patients (n = 150) who received Integrilin® had a lower incidence of mortality, MI or repeat revascularization compared to placebo (Tcheng, Harrington, Kottke-Marchant *et al.*, 1995). This was associated with an increase in minor bleeding (side effect/complication) in patients receiving eptifibatide.

Another clinical trial, the PURSUIT (Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin® Therapy) trial was a multicenter,

international, double blinded, randomised, placebo controlled study. Involving 10,948 patients, it is the largest clinical trial of any IIb/IIIa receptor antagonist drug to date (PURSUIT Trial Investigators, 1998). Integrilin® treatment demonstrated a modest 1.5% reduction in the primary endpoint measurements: death or MI within 30 days of initiation of therapy. In the U.S. and Canada, a 2.3% reduction ($p = 0.003$) in the primary endpoint measures was observed.

Because of its benefit for MI patients, and the fact that studies to determine the efficacy of Integrilin® for the treatment of cerebral embolism have not been published, Integrilin® was selected for this study.

Combination therapy

Very few studies have looked at the combination of a thrombolytic and an antiplatelet drug for the treatment of cerebral embolism, and no published studies have tested the combination of a thrombolytic with a glycoprotein IIb/IIIa receptor antagonist for the treatment of cerebral artery occlusion. The few studies that have looked at thrombolytic + antiplatelet combinations in animal models of focal ischemia are very limited in their scope because the antiplatelet agents were delivered before the embolic cerebral artery occlusion was initiated in animals (Bednar, Ouilley, Russel *et al.*, 1996; Bednar, Raymond-Russel, Booth *et al.*, 1996). In reality, treatments begin after a patient suffers a stroke, not before. Neither of the antiplatelet agents used (aspirin and ticlopidine) significantly improved the ability of tPA to lyse the clot or reduce the infarct size. The glycoprotein IIb/IIIa receptor antagonist, being a much more effective antiplatelet agent, may yield more beneficial results.

Such results have been seen in MI studies that tested the combination of glycoprotein IIb/IIIa receptor antagonists with thrombolytics. The SPEED (Strategies for Patency Enhancement in the Emergency Department) trial compared the ability of ReoPro and Retavase®, alone and in combination, to achieve TIMI 3 (thrombosis in myocardial infarction) blood flow (i.e. complete reperfusion) within 60 to 90 minutes. The group receiving the combination of the two treatments exhibited a higher incidence of TIMI 3 reperfusion within 90 minutes than either of the monotherapy groups (Anonymous: SPEED Group Investigators, 2000). Another clinical trial, the TIMI 14 (Thrombolysis in Myocardial Infarction 14) compared (among other regimens) alteplase (tPA) alone, abciximab (ReoPro) alone, and the combination of tPA and ReoPro. The primary endpoint measure was TIMI 3 blood flow at 60 and 90 minutes (Antman, Guigliano, Gibson *et al.*, 1999). The combination of the treatments yielded a higher incidence of restoration of TIMI 3 blood flow at both 60 and 90 minutes when compared with either therapy alone.

These results provide reason to believe that addition of a glycoprotein IIb/IIIa inhibitor to a thrombolytic may improve its ability to restore cerebral blood flow in patients experiencing cerebral artery occlusion. Unfortunately, in terms of drug treatments, stroke research lags far behind MI research, and so this combination has not been tested experimentally in either animal models (resembling clinical situations) or in the clinic itself.

Techniques for measuring infarct size

There are a number of methods to determine the size of the infarct in rat brain. Any method must involve differentiating the infarct from the healthy brain tissue

followed by some sort of quantitative analysis. Comparing mean infarct sizes of rat treatment groups is a powerful way of ranking the efficacy of the treatments tested (Mhairi, 1992; Hsu, 1993).

Magnetic resonance imaging (MRI) allows for visualization of an infarct in vivo. The infarction can be viewed as it evolves over time and the brain can be viewed as cross sections in any plane. Achieving a resolution of 150μm is not uncommon (van Bruggen, Thibodeaux, Palmer *et al.,* 1999). While this is without a doubt the most technically advanced method of analyzing size of the infarct, it is also the most expensive. MRI machines range in price from a few hundred thousand dollars to 5 million (http://www.varianinc.com/nmr/nmr_business.html). Another option is to pay per scan. This way, a machine does not have to be purchased, but each scan costs hundreds of dollars – still very expensive. MRI allows for visualization of infarcts as early as 30mins after the ischemic insult (Minematsu, Li, Fisher *et al.,* 1992) and is the perfect tool for studying changes in infarction over time. On the other hand, its high cost and its poor resolution relative to histological methods rendered it an unsuitable method of imaging infarcts for the purposes of this study.

Staining with hematoxylin and eosin (H & E staining) is a very common histological procedure. It is used to stain any cell type, from bone tissue to brain tissue. The resulting sections have two colors – blue due to hematoxylin and pink due to eosin. Hemotoxylin is a basic stain; thus it preferentially binds to acidic structures (e.g. nucleic acids, giving the nuclei and cytoplasmic structures containing RNA a very dark bluish black appearance). Most other cytoplasmic structures (ones that do not contain acidic molecules) preferentially bind Eosin, and appear pink. The result is a pink (light)

background dotted with blue (dark). The stark contrast between pink and blue allows for most cell structures to be seen clearly under light microscopy.

Because of the many uses for H & E staining, many different procedures exist for this type of staining, depending on the application (e.g. type of tissue being stained). H & E staining is useful for determining the extent of infarction in the brain. Infarcted areas appear lighter in color than the healthy brain tissue when H & E-stained cerebral tissue sections are viewed under a light microscope (Bederson, Pitts, Germano *et al.*, 1986). Cellular organelles (including the nuclei) rapidly disintegrate in ischemic neurons (Choi and Rotham, 1990). As mentioned, the nuclei are stained by the dark blue hematoxylin stain; therefore the disintegration of nuclei in ischemic neurons results in weaker or absent hematoxylin staining of ishemic cells. It is for this reason that the healthy tissue appears darker than the infarct.

Many studies of cerebral infarction in rodents employ H & E staining to quantify the infarct (Alexis, Dietrich, Green *et al.*, 1995; van der Worp, Bar, Kappelle *et al.*, 1998; Soonthon-Brant, Patel, Drummond *et al.*, 1999; Tsuji, Higuchi, Shiraishi *et al.*, 2000). The method involves decapitation followed by immediate removal and instant freezing of the brain. The brain is typically frozen in liquid nitrogen or methanol or any inert liquid chilled to –20°C or colder. This is done to allow for ultra-thin sectioning. A number of coronal sections, usually between 10μm and 25μm thick, are cut equidistant from each other using cryostat machinery. After these sections are stained with hematoxylin and eosin, they are viewed under a microscope and the infarct borders are marked on each slide. This allows the calculation of infarct area for each section (with the help of software). The infarct area of a given section is multiplied by the distance between adjacent sections to yield an infarct volume. The sum of the infarct volumes (of all the

sections) represents the estimated total infarct volume of the brain. A greater number of coronal sections results in a more accurate estimate of the infarct volume. This is an accurate but time-consuming method of quantifying the infarction in rodent models of cerebral ischemia.

The tetrazolium salt 2,3,5-triphenyltetrazolium chloride (TTC) is used to determine the presence of an infarct in many different tissue types, including cerebral infarcts. The colorless tetrazolium ions are reduced by dehydrogenase enzymes (e.g. succinate dehydrogenase) of the mitochondria to pigmented formazan (Nachlas, Tson, Souza *et al.*, 1957). TTC is therefore a mitochondrial stain; only those cells with functional mitochondria take up the red stain and become red. The dead brain tissue (lacking mitochondria) appears white. The total staining procedure, including removal of the brain, slicing, and staining takes less than 30mins, making it a very convenient method of differentiation of the infarct from healthy brain tissue in coronal cross sections.

This technique is acceptable when the state of the mitochondria accurately reflects the state of the cell. If functional mitochondria exist in cells whose nuclei have lysed, the cell will still stain. For this reason, TTC can be used to accurately measure infarction in rat brains a minimum of 24hrs post occlusion (Hatfield, Mendelow, Perry *et al.*, 1991; Isayama, Pitts, Nishimura, 1991).

The three methods described, TTC staining, H & E staining, and MRI imaging are all accurate methods of measuring the infarct size. This is strongly supported by the high correlation observed between these methods when they are used to study a given sample. The following table shows the observed correlation coefficient, r, when these three methods were compared with each other for measurement of infarct size (Barone, Clark, Feuerstein *et al.*, 1991):

Table 1: Correlation coefficients of infarct size-calculation methods

Methods of calculating infarct size	MRI vs. H & E	MRI vs. TTC	H & E vs. TTC
Correlation coefficient (r)	0.95	0.94	0.98

Of the techniques discussed, TTC staining is the cheapest and most convenient, and its accuracy is demonstrated by the high correlation with the H & E method (the most accurate method). After the coronally cut brain slices are immersed for 20mins and scanned, and the infarct size can be calculated within minutes. This is in contrast to the hours required to achieve these results using H & E. For these reasons, TTC staining was chosen for visualizing the infarcted tissue.

Objective and hypothesis

To date, the only effective therapy for acute embolic stroke is thrombolytic therapy with tissue plasminogen activators (Albers, 1999; Benavente and Hart, 1999; Traynelis and Lipton, 2001). The effectiveness of thrombolytic therapy is limited by the dose-dependent risk of hemorrhagic transformation. For this reason, 90-95% of stroke patients do not qualify for thrombolytic therapy. If supplementing a thrombolytic with an antiplatelet medication allows for effective clot lysis at lower doses of thrombolytic, we may reduce the risk of hemorrhagic complications associated with thrombolysis. This may also allow a greater portion of stroke patients to qualify for thrombolysis.

The purpose of the project described in this thesis was to determine if combination therapy with Integrilin® (the glycoprotein IIb/IIIa antagonist used in our lab) and Retavase® is more effective than either therapy alone. If this is the case, then this combination may allow for a lower effective dose of rPA in the clinical setting, resulting

in a decreased incidence of rPA-induced hemorrhagic transformation. This approach can save lives and reduce disability in stroke patients and possibly result in more relaxed eligibility criteria for thrombolytic + glycoprotein IIIa antagonist treatment (compared with thrombolytic treatment alone).

Chapter 2: Methods

Assignment of rats to groups

Male Wistar rats (350g to 400g) purchased from Charles River (St. Constant, Canada) were used. They were housed in a 12hr light:dark cycle and were given free access to food and water at all times. The experiment was carried out with the permission of and in accordance with the guidelines set by the Health Sciences Animal Ethics Committee of the University of Alberta.

Ischemic rats are those that received a clot as described in *Introduction of an embolus into the MCA*. Control rats underwent the same surgical procedure as the ischemic rats except that the rats' own blood (not a preformed thrombus) was injected into the base of the MCA. Ischemic and control rats were randomly assigned to one of four treatment groups: rPA only, Integrilin® only, rPA + Integrilin®, and no treatment. One to four rats were assigned to each of the control groups. In the ischemic groups, analyses were not considered for those rats that did not survive 72hrs post infarction. Of the rats that did survive 72hrs, only those rats that showed signs of infarction (behaviour deficit and/or positive TTC result) were considered for analysis. A minimum of seven rats were analyzed per group.

Preparation of the catheter

The catheter consists of two pieces of polyethylene (PE) tubing glued together. The tubing was purchased from Becton Dickinson & Co., New Jersey, USA. The (large) PE-50 tubing has an outside diameter of 0.965mm and an inside diameter of 0.58mm. It attaches to the syringe and holds the bulk of fluid (i.e. blood, thrombin or saline). The

(small) PE-10 tubing has an outside diameter of 0.61mm and an inside diameter of 0.28mm. This is the tubing that is introduced into the CCA and advanced to the MCA as will be described later. The PE-10 tubing is heated until it softens so that it can be stretched so that the outside diameter is reduced to 0.25mm. Tubing thicker than 0.3mm will likely become stuck in the ICA before it reaches the MCA. After thinning, the modified PE-10 tube is cut so that one tip is 0.25mm diameter and the other tip retains its original diameter. The thick side is inserted into the PE-50 tube and glued into place, so that 17mm of the PE-10 tube remains exposed. The PE-50 tubing used in this experiment was 3.5cm long (figure 2.1).

Introduction of an embolus into the MCA

I tested my hypothesis by using the rat model of embolic MCA occlusion that will be described. The following surgical procedure was conducted on anesthesized male Wistar rats (350 to 400 grams).

Injection of a clot in the right middle cerebral artery (rMCA) presents a challenge because the MCA cannot be accessed directly. Instead, a catheter filled with bovine thrombin (purchased from Thrombostat, TM Warner-Lambert Co., Scarborough, Canada) is advanced 17mm up the right internal carotid artery (rICA), so that the tip of the catheter is in close proximity to the rMCA (within 2mm). All arteries referred to will be on the right side of the body.

Figure 2.1: Modified PE-50 catheter

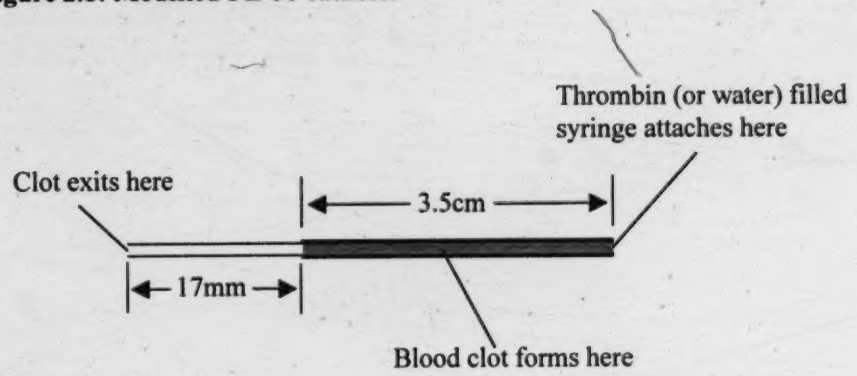

Figure 2.1
The clot is formed in the PE-50 tubing - a length of 3.5cm was used in this experiment, corresponding to a volume of ~10µl. The length of the PE-50 tubing affects the volume of blood used to form the clot, and thus affects the size of the resulting infarct. Only the PE-10 tubing (17mm) enters the ICA.

The rats receive a 70:30 nitrous oxide:oxygen mixture throughout the procedure. Anesthesia is induced with 2.5% halothane (purchased from MTC Pharmaceuticals, Cambridge, Canada) for 12-15mins and maintained with 1.5% halothane in this gas mixture. In addition to this, the breathing rate and depth are used to determine the correct halothane setting (e.g. if breathing becomes shallow, the halothane setting is lowered). An incision is made in the neck and the CCA (common carotid artery), ECA (external carotid artery), and ICA are exposed by removing as much soft tissue, nerves, membranes, etc. from these arteries as possible while avoiding unnecessary damage.

The CCA divides into the ECA and ICA (figure 2.2). The arteries are exposed at the point of bifurcation. As mentioned, a catheter will be advanced up the ICA, but the point of entry will be the ECA, not the ICA. An incision in a large carotid artery cannot be closed, and the only way to prevent bleeding after the surgery is to seal the artery at both sides of the incision (figure 2.3). Blood flow through the ICA enters the brain and the MCA (figure 2.4), so tying this artery would be lethal to the rat. On the other hand, the ECA does not irrigate the brain.

The fact that the ICA and ECA both branch off in the same direction presents an obstacle because a catheter introduced into the ECA will traverse down the CCA and not up the ICA as desired (figure 2.2). To circumvent this problem, the ECA is tied shut at two points and cut in between those points. The ~6mm of ECA still attached to the junction is moved so that insertion of a catheter in the ECA would result in its travel up the ICA and not down the CCA (figure 2.5).

Figure 2.2: Carotid arteries of the neck

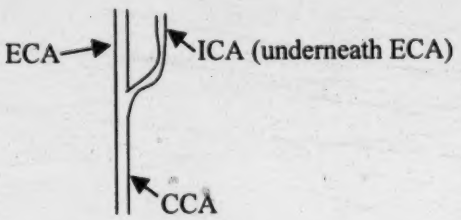

Figure 2.2
The CCA divides into the ECA and ICA. At first, only the ECA and CCA are visible because the ICA is underneath the ECA. After ligating and cutting the ECA, and removing some more tissue, the ICA is exposed.

Figure 2.3: Sealing an incision in a carotid artery

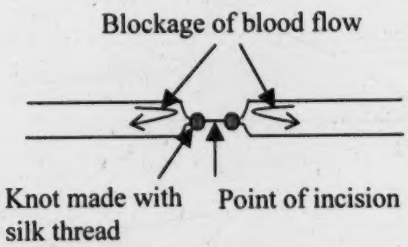

Figure 2.3
The ECA is ligated at two points and cut in between.

Figure 2.4: The main arteries in the rat cerebral circulation

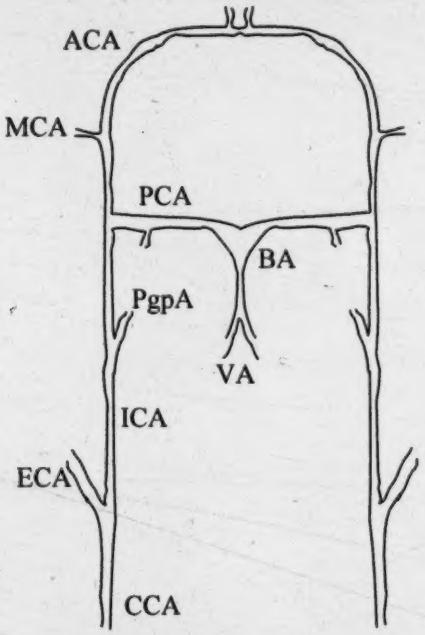

Figure 2.4
The main arteries in the rat cerebral circulation, illustrating the circle of Willis. The right side of the circulation appears on the left side of the diagram, as this is a ventral view. The vertebral arteries (VA) merge (posterior circulation), forming the basilar artery (BA). The anterior circulation includes the CCA and ICA, off of which the MCA and ACA branch. A catheter cannot be introduced into the ICA because after it is removed, sealing the hole would compromise blood flow to the brain. If the rCCA is clamped, blood from the the left side of the circulation may enter the right side of the circulation via the circle of Willis. The cerebral circulation begins anterior to the pterygopalatine artery.

Figure 2.5 Aligning the ECA with the ICA

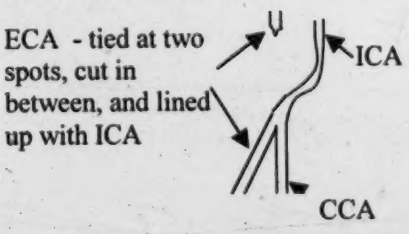

Figure 2.5
Lining up the ECA with the ICA is essential to so that introduction of the catheter into the ECA will result in its entering the ICA, not the CCA. To line them up, the ECA must be cut first.

A hole must be made in the ECA to allow for entry of the catheter. However, making an incision in the ECA at this point would result in heavy bleeding. To prevent this, the CCA and ICA must *both* be clamped. If only the CCA is clamped, blood can travel from the left to the right side of the circulation at a number of points downstream where the two circulations meet and travel retrograde down the ICA and bleed out of the incision in the ECA (figure 2.4). That is why the ICA must also be clamped. Before making an incision in the ECA, the portion of the ECA distal to the incision (i.e. closer to the junction) is surrounded by a silk thread such that tugging on the thread would press the walls of the ECA on the catheter inside it.

After the CCA and ICA are clamped with microvascular clamps, an incision is then made on the ECA. A catheter is inserted into this incision and advanced into the ECA until it reaches the junction. From there it is advanced up the ICA until it meets the clamp on the ICA. Removal of the clamp on the ICA at this point will cause blood to flow around the catheter and out the incision, so the silk thread on the ECA (mentioned at the end of last paragraph) is tugged gently. The resulting knot presses the walls of the ECA against the catheter, preventing blood from flowing around it. This knot must not be too tight or the catheter will not be able to slide through the ECA.

At this point, the clamp on the ICA is removed and the whole 17mm length of catheter is advanced up the ICA. The ptyergopalatine artery (PgpA), which branches off of the ICA, is pinched with an artery forcep to ensure that the catheter does not stray into this branch. The catheter and the 100μL Hamilton syringe (attached to the tubing) contain either bovine thrombin (ischemic rats) or saline (non-ischemic controls/sham surgery). Blood is withdrawn until it fills the whole length of tubing. After waiting for 15mins (to allow thrombus formation in the catheter), the thrombin-filled syringe is

switched with a saline-filled 100 μL Hamilton Syringe and the clot is injected slowly. The CCA remains clamped and the catheter remains inserted for another 15mins after clot injection to allow the clot to settle in the MCA. The catheter blocks some collateral blood flow (figure 2.6), preventing the clot from washing away from the MCA. The retrograde blood pressure in the ICA attempts to force the clot backward. The catheter blocks it, leaving the blood and the clot with only one way to travel, up the MCA (figure 2.6). So the CCA must remain clamped for 15mins after injection of the clot to maintain the retrograde force exerted on the clot.

After this delay, the catheter is removed and the loose knot on the ECA is tightened immediately after to prevent bleeding from the incision. The clamp is removed from the CCA and the surgical wound is closed with skin staples. The rat is allowed to recover from anesthesia.

Figure 2.6: Embolic MCA occlusion

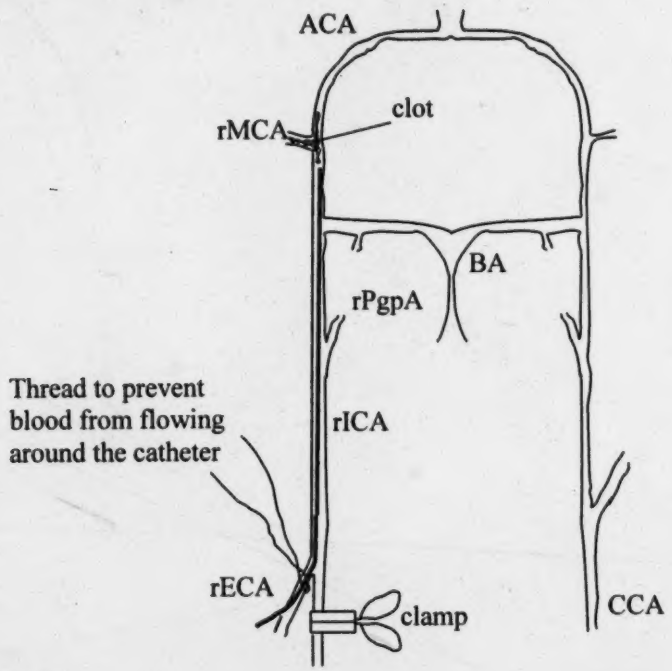

Figure 2.6
The catheter is advanced up the rICA, bypassing the PgpA, to within 2mm of the rMCA. The rCCA is clamped. The clot is injected as a stringy platelet aggregate. Blood from the left side of the circulation can traverse through the MCA (and carry the clot with it), but not down the ICA because the catheter blocks that route. Upon MCAo, blood from the left side of the circulation exerts a force on the clot, pushing it firmly into the MCA.

Intravenous injection of drug(s) via the tail vein

Rats are anesthetized again with 3% halothane for 2-5mins in the 70:30 nitrous oxide to oxygen air mixture. The halothane concentration is reduced to 1.5% for a few minutes, then to 1.0% for the remainder of the infusion.

Making an incision (1.5cm to 2cm) along the length of the tail (ventral side) exposes the tail vein. Due to collateral circulation, an incision can only be made by clamping the vein at two points and cutting in between those points. After this is done, a catheter is inserted into the tail vein – the tip faces the direction of blood flow. This catheter is attached to a drug pump that can be programmed to infuse the drug at a given rate and time period. One clamp is removed to allow the catheter to advance all the way in. A silk thread is tied around the vein-wall so that it is snug against the catheter (to prevent back-flow of drug and/or blood past the catheter). The infusion is allowed to begin after this.

The part of the vein held by the other clamp is tied shut (with silk thread) and the clamp is removed. After the infusion is completed, the catheter is removed from the vein and the thread (that was keeping the wall of the vein against the catheter) is tightened to seal that part of the vein also. The wound is closed with a Band-Aid.

Drug infusion was initiated 1hr after the clot injection. Integrilin® was infused for 2hrs at a rate of 0.36mg/kg/hr after a bolus of 0.18mg/kg in the Integrilin® only group (unpublished data from our laboratory revealed this to be a good protocol for Integrilin®). Retavase® was given as 2 equal boluses delivered 30mins apart. Four doses were tested in the Retavase® only group: 0.5mg/kg, 1.0mg/kg, 1.5mg/kg, and 2.0mg/kg - each bolus was half of the mentioned dose (e.g. 0.5mg/kg dose was given as 2 boluses of 0.25mg/kg). In the combination therapy group, infusion of Integrilin® was initiated after

cessation of rPA and continued for 2hrs. The dose of Integrilin® given to the rats in the combination therapy group was the same as in the Integrilin® only group (0.18mg/kg bolus, 0.36mg/kg/hr for 2hr). Two doses of Retavase® were tested in the combination therapy group: 0.5mg/kg and 1.0mg/kg. Retavase® was administered as a double bolus (30mins apart) in the combination therapy group.

Variables measured

Neurological deficit was measured on a 5 point scale: 0 = no deficit, 1 = forelimb flexion, 2 = reduced resistance to lateral push, 3 = unilateral circling, 4 = unilateral circling with decreased consciousness. In the no treatment group, neurological deficit (ND) was measured at 2hrs and 48hrs after clot (or blood injection).

During the one hour between clot injection and initiation of drug infusion, attempts were made to measure the ND score, but this was not always possible as some rats remained under the effect of anesthesia during this time. ND was also measured 2hrs after termination of drug infusion and 48hrs post occlusion (or blood injection). Ischemic rats that did not show ND at any point in time after artery occlusion (and therefore had no infarction when sacrificed 72hrs later) were not included in the analysis. In all likelihood, MCAo did not take place in these rats. About 15% of ischemic rats in each group (including controls) were excluded from the analyses for this reason.

After 72hrs, the rats in each group were anesthetized and sacrificed by decapitation. Their brains were removed and immediately rinsed in ice-cold saline (0.5%) and sliced into 2mm coronal sections in a brain block – the brain block was partially immersed in ice-cold water. The brain slices were immersed in a 2% TTC solution and incubated for 20mins. The slices were flipped and scanned on a flatbed

scanner. The images were analyzed using Adobe Photoshop (Yang, Li, Shuaib, 1998). The white areas (infarction) were traced using a mouse (freehand) and the number of pixels were calculated by pressing an action key (F6). The total right hemisphere areas were also measured using this method by outlining the entire hemisphere (freehand). For each rat, the total number of white pixels (in all right hemisphere slices) were divided by the total number of pixels (in all right hemisphere slices). This value was multiplied by 100% to calculate the percentage infarction of the right hemisphere.

Finally, the number of rats that died before 72hrs post occlusion was recorded for each group. All rats, those that were sacrificed at 72hrs post occlusion and those that died before 72hrs post occlusion, were examined for cerebral hemorrhage.

Similarities and differences of infarct size between the groups were analyzed using the one-way Analysis of Variance (ANOVA) option on Microsoft Excel. Use of the extra sum of squares F-test allowed for multiple comparisons between the treatment groups. A large number of rats per group (30 or more) would be necessary to compare binomial variables such as incidence of mortality or hemorrhagic transformation. Despite the fact that safe statistical analyses, confirming significant differences between groups, cannot be made for comparisons of mortality rate and incidence of fatal hemorrhagic transformation, these data were included because this information is important. Furthermore, the trends observed for these variables provide justification for larger scale studies to examine them more accurately.

Chapter 3: Results

Nonischemic controls

Rats that underwent sham surgery (saline was used in place of thrombin) did not show any signs of ND or infarction. None of these rats experienced cerebral hemorrhage. One rat in the high dose (2.0mg/kg Retavase® group) died after receiving the drug. No trace of bleeding was found in the brain; therefore the cause of death for that rat is unknown.

Table 2: Results for Non-Ischemic Controls

Group	# of rats	Mean ND after occlusion	Mean ND at 48hrs	Mean infarct %	Incidence of Hemorrhagic transformation	Mortality
No treatment	5	0	0	0	0	0
Integrilin®	4	0	0	0	0	0
0.5mg/kg Retavase®	4	0	0	0	0	0
1.0mg/kg Retavase®	1	0	0	0	0	0
1.5mg/kg Retavase®	1	0	0	0	0	0
2.0mg/kg Retavase®	3	0	0	0	0	1
0.5mg/kg Retavase® + Integrilin®	1	0	0	0	0	0
1.0mg/kg Retavase® + Integrilin®	1	0	0	0	0	0

In the interest of conservation, fewer rats were used in the non-ischemic control groups. The purpose of conducting sham surgeries was simply to demonstrate that the ND, mortality, cerebral hemorrhage, and cerebral infarction were due to the clot being injected

into the brain and not some other factor. For this reason, it was not necessary to use a large number of rats per group. If, for example, the clamping of the CCA was responsible for the neurological damage (by reducing blood flow to the brain), then the thrombolytic or antiplatelet drugs would not be expected to be beneficial. By performing the sham surgeries, we were able to rule out this and other such possibilities, which will be covered in the discussion.

Infarct sizes in ischemic rats

Infarct percentages were measured from those rats that survived 72hrs after injection of the embolus. Control rats had the largest infarcts. Infarct perventages for the 1.5mg/kg and 2.0mg/kg Retavase® groups and the 1.0mg/kg rPA + Integrilin combination group are not shown because not enough rats survived 72hrs in those groups to involve them in meaningful statistical analyses (figure 3.1).

Figure 3.2 shows scanned images of rat brain slices. The image on the left was taken from a non-ischemic control rat. The image on the right, taken from an ischemic animal, shows the infarct tissue (white).

Figure 3.1: Infarct percentage vs. treatment

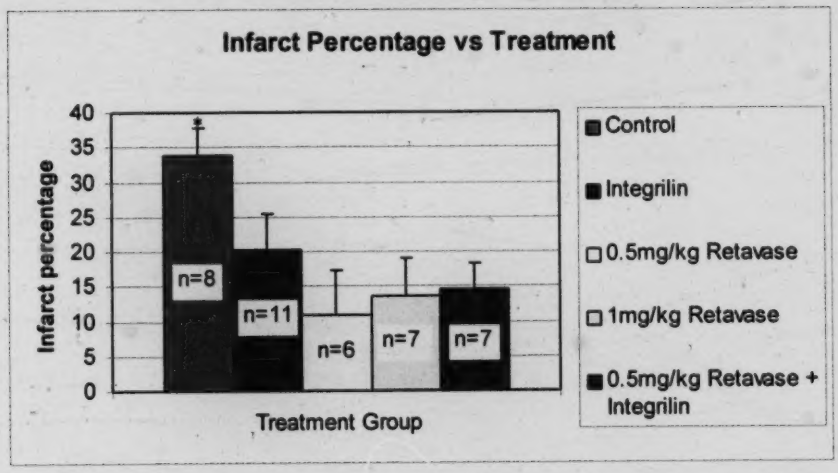

Figure 3.1
Infarct sizes were measured as the percent of right hemisphere occupied by the infarction. Error bars represent one standard error (of the mean). Ischemic control rats had significantly higher infarct sizes than all treatment groups. There were no statistically significant differences in the infarct size between any of the treatment groups.

Figure 3.2: Scanned images of 2mm thick coronal rat brain sections

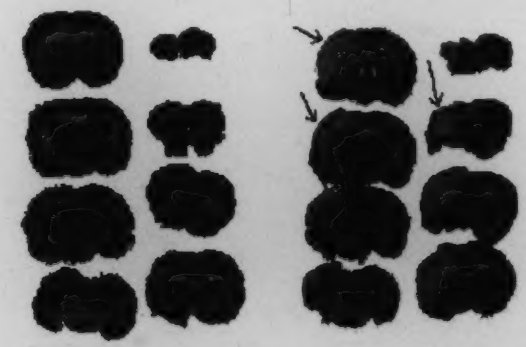

Figure 3.2
TTC-stained rat brain slices from a sham-operated rat are on the left and those from an ischemic rat are on the right. Healthy tissue appears darker (red in a color image). The infarction appears paler (white in a color image). Arrows point to areas of infarction. The infarct size is expressed as sum of the white areas of all slices divided by the sum of the ipsilateral total hemispheric areas.

An Analysis of Variance (ANOVA) F-test performed with the full model (5 separate means illustrated in figure 3.1) vs. the reduced model (1 mean for all 5 groups) revealed an F-statistic of F=3.11 which corresponds to a p-value of p=0.027. This suggests that at least one of the mean infarct sizes in the 5 groups subjected to the ANOVA test is different. An Extra Sum of Squares F-test comparing the full model (5 separate means) vs. the reduced model (2 means, 1 for control, 1 for the remaining 4 treatment groups) revealed an F-statistic of F=0.68 which corresponds to a p-value of p=0.57. Therefore, none of the mean infarct sizes in the drug treatment groups is significantly different from each other. The Extra Sum of Squares F-test was performed with the full model (2 means, 1 for control, 1 for the remaining 4 treatment groups) vs. the reduced model (1 mean for all 5 groups). This revealed an F-statistic of F=10.67 corresponding to a p-value of p=0.002. This is strong evidence that the control group had a significantly higher mean infarct size than all of the drug treated groups. The ANOVA Table is summarized below:

Table 3: ANOVA for 4 treatment groups and control group

Source of variance	Sum of squares	Degrees of freedom	Mean square	F-statistic	p-value
Between groups	2602.885	4	650.721	3.112	0.027
Control vs. treatments	2174.856	1	2174.856	10.669	0.002
Among treatments	428.029	3	142.677	0.682	0.569
Within groups	7318.204	35	209.091		
Total	9921.089	39			

Neurological Deficit Scores in Ischemic Rats

A useful measure of drug effect is the actual decrease in ND scores after the treatment compared to control rats. All treatments resulted in greater decreases in ND score than control rats. The greatest decrease was seen in the 0.5mg/kg Retavase® group.

Figure 3.3 shows the mean ND score calculations for the treatment groups at 2hrs after MCAo (data point on left) and 48hrs after MCAo (data points on right). Comparing the slopes is a rough way to compare the effectiveness of the different treatments (greater slope = more effective). Figure 3.4 shows the same information as figure 3.3 except that the differences between the individual groups are more visible in figure 3.4.

Figure 3.3: Change in neurological deficit score over time

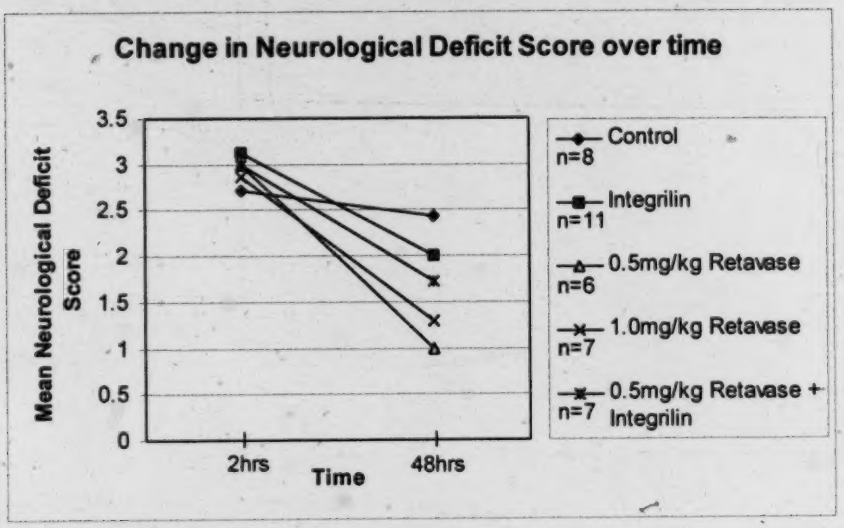

Figure 3.3
A greater decrease in neurological deficit scores was observed in treated rats than in the ischemic control rats.

Figure 3.4: Decrease in neurological deficit score over 48hrs

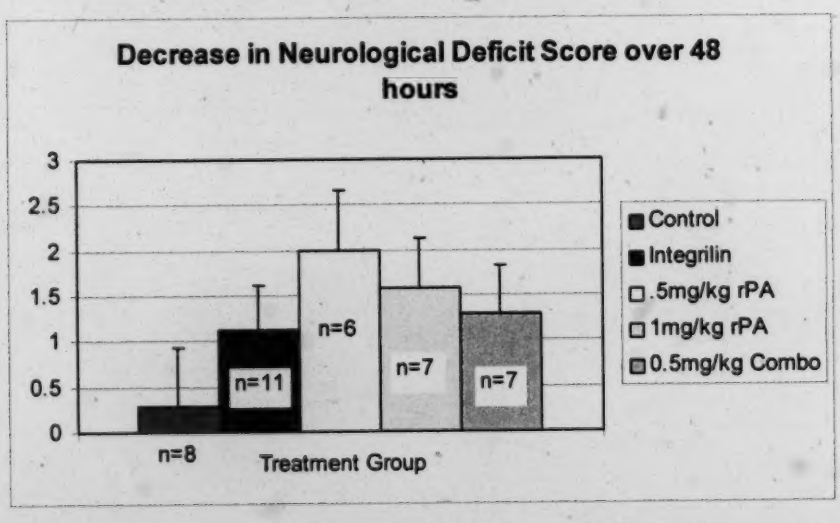

Figure 3.4
The control group shows an almost negligible decrease in neurological deficit score whereas the treatment groups show a pronounced therapeutic effect. Error bars represent standard error of the mean.

As with the infarct percentage calculations, these measurements are presented only for those rats that survived 72hrs post occlusion. It should be noted that all rats that survived 48hrs lived onto 72hrs (except one, which died 50hrs after artery occlusion). Drug treatments did not affect ND scores right away. The drug effect (i.e. reduction in ND scores) was much more prominent when measurements were taken 48hrs after the treatment. The following data table shows all the mean ND scores for all groups taken at both time points.

Table 4: Neurological Deficit Scores

Control	2hrs post occlusion	2.7
	48hrs post occlusion	2.1
Integrilin® Only	40mins post occlusion	2.9
	2hrs post infusion	2.9
	48hrs post occlusion	1.9
0.5mg/kg Retavase®	40mins post occlusion	3.0
	2hrs post infusion	3.0
	48hrs post occlusion	1.5
1.0mg/kg Retavase®	40mins post occlusion	3.0
	2hrs post infusion	3.0
	48hrs post occlusion	2.2
*1.5mg/kg Retavase®	40mins post occlusion	3.0
	2hrs post infusion	3.0
	48hrs post occlusion	2.0
*2.0mg/kg Retavase®	40mins post occlusion	3.0
	2hrs post infusion	2.5
	48hrs post occlusion	1.5
1.0mg/kg Retavase® + Integrilin®	40mins post occlusion	3.0
	2hrs post infusion	3.0
	48hrs post occlusion	2.3
*0.5mg/kg Retavase® + Integrilin®	40mins post occlusion	3.0
	2hrs post infusion	3.0
	48hrs post occlusion	1.5

* No more than 4 rats survived these treatments at 72hrs post occlusion

The groups that were omitted from the infarct percentage analysis were also omitted from the analysis of ND scores (for the same reason) because there were not enough survivors in those groups to allow for a meaningful statistical analysis.

The validity of taking ND scores was tested by correlating the mean ND scores (at 48hrs) with the infarct size for the control and treatment groups. The calculated correlation coefficient, r, was equal to 0.916. This high value shows a strong linear relationship between infarct size and ND scores in these animals. This is also demonstrated in figure 3.5.

Figure 3.5: Infarct size vs. neurological deficit score scatterplot

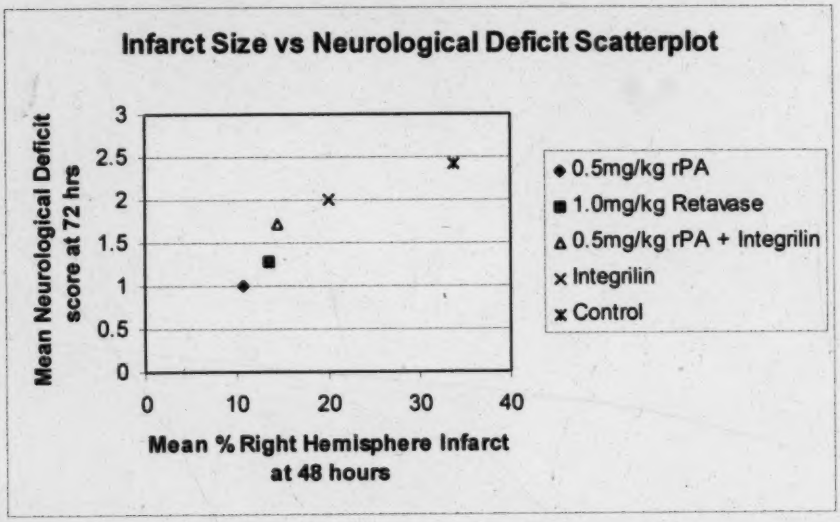

Figure 3.5
Correlating the mean neurological deficit scores with mean infarct percentages. The neurological deficit scores recorded at 48 hours port-occlusion were averaged for the control group (✱), the Integrilin® only group(×), the 0.5mg/kg (▲)and 1.0mg/kg (■) Retavase® only groups, and the 0.5mg/kg Retavase® + Integrilin® combination therapy group(◆). These values were correlated with the mean infarct sizes of the rats in those groups.

Incidence of Hemorrhagic Transformation in Ischemic Rats

A side effect of thrombolytic and antiplatelet drugs is bleeding. This is immediately noticeable at the wound site on the tail through which the catheter enters. Hemorrhagic transformation in the brain was almost always fatal (one exception, in the Integrilin® group). Fatalities due to hemorrhagic transformation were observed for Retavase®, but not with Integrilin®. The risk of hemorrhagic transformation appeared to be proportional to the dose of Retavase® administered in the monotherapy and combination therapy groups.

The Integrilin® alone group and the combination group with 0.5mg/kg Retavase® had no rats die of cerebral hemorrhage, and that is why they are not visible on the graph. The Integrilin® alone group had one rat with intracerebral hemorrage that was noticed during TTC staining, but it survived 72hrs, and was therefore not classified as a fatal hemorrhagic transformation.

Figure 3.6: Percentage of fatal hemorrhagic transformation per group

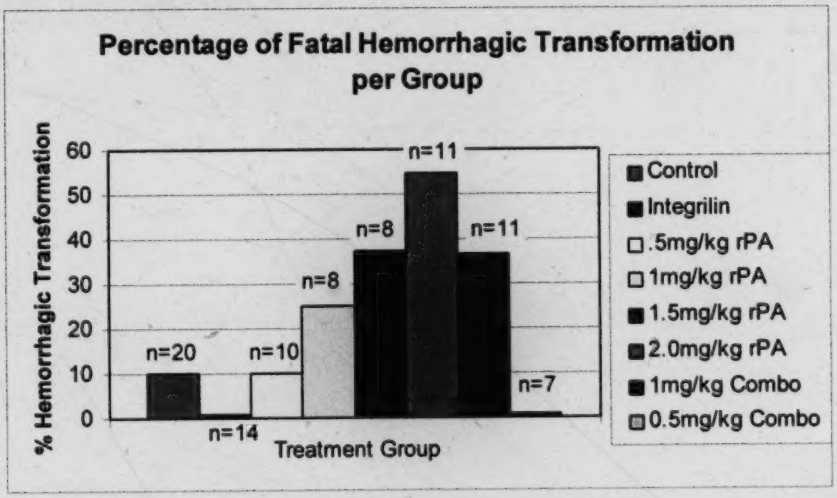

Figure 3.6
Increasing the dose of Retavase® increased the incidence of fatal hemorrhagic transformation and therefore eliminated any benefit derived from lysis of the embolus. Supplementing 1.0mg/kg Retavase® with Integrilin® increased the risk of hemorrhagic transformation. This was rectified by lowering the dose of Retavase® in the combination group to 0.5mg/kg. At this dose, hemorrhagic transformation was not observed. Statistical analyses cannot be performed on binomial variables unless group sizes are large (n>30 per group).

Mortality in Ischemic Rats

The reductions in ND scores, infarct size, or incidence of hemorrhagic transformation seen with various treatment strategies mean nothing unless there is a significant decrease in mortality associated with the treatment. The following graph shows overall mortality for each group, without specification of the cause of mortality. The greatest mortality was seen in three groups: the control, the 2.0mg/kg Retavase® group, and the combination therapy group with 1.0mg/kg Retavase®. The latter two groups had high mortality due to hemorrhagic transformation in the brain whereas almost all control rats died because of the embolic artery occlusion. The groups with least mortality were the Integrilin® alone and the combination group with 0.5mg/kg Retavase®.

Figure 3.7: Percentage mortality per group

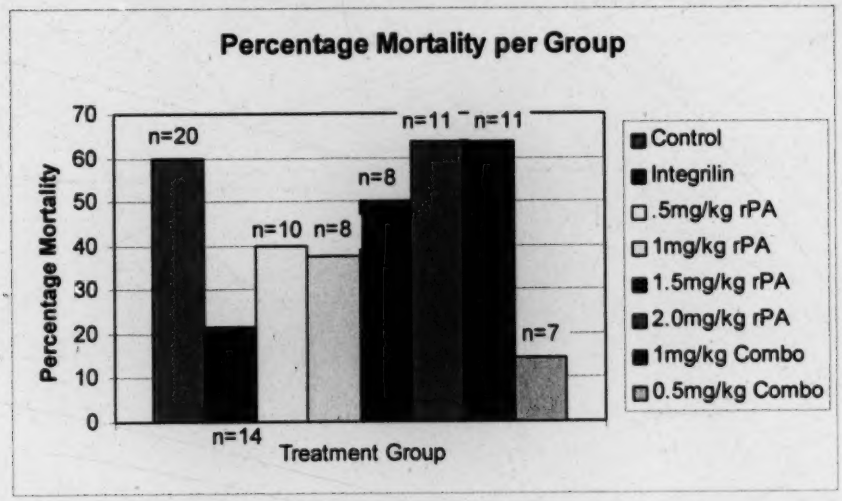

Figure 3.7
Lowest mortality was observed the combination therapy group with 0.5mg/kg Retavase. The Integrilin monotherapy groups exhibited a lower mortality rate than any of the Retavase monotherapy groups.
Statistical analyses cannot be performed on binomial variables unless group sizes are large (n>30 per group).

Chapter 4: Conclusions

The benefits of early reperfusion

All drug-treated rats showed significantly smaller infarct volumes and significant reductions in ND scores at 72hrs post infarction when compared with control (non-treated) ischemic rats. Both Integrilin® and Retavase® contribute to clot dissolution, so the most reasonable explanation for these drug effects is that by degrading the embolus, the drugs facilitated the restoration of cerebral blood flow, allowing some brain tissue to be saved from ischemic injury. This would account for the lower infarct size and reduced ND scores observed in the drug-treated groups.

Whereas cerebral reperfusion is achieved during the drug administration, the decrease in ND score was not observed until several hours after the drug was given. This phenomenon is not inconsistent with the idea that early reperfusion explains the decrease in ND scores and infarct sizes seen with the drug-treated rats. Monkeys with mechanical MCA occlusion took 3hrs before NDs began to disappear after reperfusion of the MCA (Garcia, Mitchem, Briggs *et al.*, 1983).

It is possible that the neurons that benefited from the drug treatments were barely functional immediately after reperfusion, and took time to regenerate their intracellular biochemistry and return to normal function. Only after the affected tissue regains its function may we detect improvement in the motor abilities measured for determination of the ND score. This idea is supported by an experiment which showed that if ischemia lasted longer than 30mins, even 4hrs after reperfusion, ATP levels were only 80% of control (Sun, Zhang, Lin *et al.*, 1995).

The results of this experiment support the theory that early reperfusion in patients suffering embolic stroke reduces the size of the infarct and the resulting disability.

Mortality due to infarction vs. mortality due to hemorrhage

Integrilin® binds to glycoprotein IIb/IIIa receptors and prevents platelet aggregation and thus prevents clot formation (Tcheng, 1996). Retavase® causes cleavage of fibrin molecules, causing clots to break up. In the event of bleeding, either at the wound site, or intracerebrally (or anywhere for that matter), the ability to stop the bleeding via coagulation of the blood is severely impaired in rats receiving either Integrilin®, Retavase®, or the combination of these drugs. The impaired ability of the blood to clot combined with ischemia-induced degradation of the blood-brain barrier is the probable reason for the increased risk of intracerebral hemorrhage during administration of anticoagulants, antiplatelet drugs, or thrombolytics. This would explain the dose-dependent (dose of Retavase®) risk of fatal hemorrhagic transformation in rats receiving Retavase® monotherapy and combination therapy. It is interesting to observe that while 1.0mg/kg Retavase® alone caused 25% fatal hemorrhagic transformation, the combination of 1.0mg/kg Retavase® with Integrilin® resulted in a 36% rate of fatal hemorrhagic transformation. The lower dose, 0.5mg/kg of Retavase®, was therefore a better choice for combination with Integrilin®.

While the low (0.5mg/kg) dose of Retavase® (monotherapy) appeared to show the greatest decrease in ND score and the lowest infarct volumes in those rats that survived 72hrs post ischemia, these parameters alone do not tell the whole story. The primary endpoint measure used in clinical drug trials of stroke or MI is mortality – in this experiment, it is mortality at 72hrs post ischemia. The group demonstrating the lowest

mortality rate was the combination therapy group with 0.5mg/kg Retavase® and Integrilin®. It may seem paradoxical that this treatment would result in the lowest mortality rate without showing similar superiority in its ability to reduce ND scores and infarct sizes when compared to other treatments. It is reasonable to assume that those ischemic rats with infarct sizes larger then a critical size end up dying before 72hrs and are therefore not included in infarct size analyses. If a drug can prevent infarcts (in some rats) from growing that large, a number of rats that would have died to due excessive infarction may be saved by drug treatment and included in the infarct size analyses in those drug groups. Thus, the difference in rat survival between groups may introduce a selection bias by filtering out severely infarcted rat brains in poor/less effective treatment groups only. It is reasonable to hypothesize that those rats that would have ended up dead in other drug treatment groups (due to severity of the infarct) were salvaged in the combination group. In other words, increased mortality may be representative of higher infarct sizes whereas low mortality indicates lower infarct sizes. The fact that the combination group exhibited the lowest mortality rate with a mean infarct size comparable to other treatment groups suggests that it was the best treatment.

The combination therapy group with low dose Retavase® showed no incidence of hemorrhagic transformation and the lowest mortality rate. Thus the combination therapy appears to be the best treatment for our rat models of embolic MCA occlusion.

Implications of these Results

These results provide justification for a larger scale study with more valid (and more expensive) models of human MCAo. The greatest benefit of antiplatelet treatment is in the prevention of reocclusion (Saito, Saitoh, Asakura *et al.*, 1993; Willerson, Golino,

McNatt, 1991) and for this reason it is administered for days, not hours. With the rat, the kinetics of clot dissolution is much faster than in larger species like rabbits, monkeys, and humans. In almost all rats, there is no trace of the embolus 3hrs after injection into the MCA (Wang, Todd, Yang *et al.*, 2001) – it is likely dissolved by the rat's endogenous clot lysis systems. This limits our ability to test thrombolytics and antiplatelet drugs at different time points after artery occlusion because if the clot has dissolved by 2-3hrs, there will be no clot for the drug to act on. It is therefore plausible that the combination therapy treatment would show more benefit, relative to monotherapy groups, in larger species than in the rat.

The incidence of hemorrhagic transformation may be reduced by lowering the dose of thrombolytic drug administered. Combination therapy may allow for reduced doses of thrombolytic agent in human stroke patients while maintaining its efficacy at dissolving the embolus. This strategy has the potential to make thrombolysis a safer treatment, allowing a greater percent of patients to benefit from thrombolytic therapy in the future and for fewer cases of treatment-related intracerebral hemorrhage.

While the Integrilin® alone therapy was not as effective in preventing mortality as the combination group, it was a very safe treatment, demonstrating 0% incidence of fatal hemorrhagic transformation. As a result, the Integrilin® monotherapy group demonstrated a lower mortality rate than any of the Retavase® monotherpy groups. Integrilin® may be safe to administer in situations where thrombolytics are not. Integrilin® monotherapy has demonstrated beneficial results in MI patients but has not been tested in embolic stroke patients. Thus Integrilin® monotherapy should be studied in stroke patients as a potential alternative to thrombolytic therapy. While it may not be

the best therapeutic option, glycoprotein IIb/IIIa antagonist monotherapy may prove to be a more flexible treatment than the currently used thrombolytic therapy.

Chapter 5: References

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